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Localization-Dependent and -Independent Roles of Numb Contribute to Cell-Fate Specification in *Drosophila*

Sheetal Bhalerao,[1,2] Daniela Berdnik,[2,3] Tibor Török,[2,4] and Juergen A. Knoblich[1,*]
[1]Institute of Molecular Biotechnology of the Austrian Academy of Sciences (IMBA)
Dr. Bohr Gasse 3-5
1030 Vienna
Austria
[2]Research Institute of Molecular Pathology (IMP)
Dr. Bohr Gasse 7
1030 Vienna
Austria
[3]Department of Biological Sciences
Stanford University
Stanford, California 94305
[4]Biological Research Center of the Hungarian Academy of Sciences
Institute of Genetics
H-6701 Szeged
P.O.B. 521
Hungary

Summary

During asymmetric cell division, protein determinants are segregated into one of the two daughter cells [1]. The Numb protein acts as a segregating determinant during both mouse and *Drosophila* development [2, 3]. In flies, Numb localizes asymmetrically and is required for cell-fate specification in the central [4] and peripheral nervous systems [2, 3], as well as during muscle [5, 6] and heart [7] development. Whether its asymmetric segregation is important to the performance of these functions is not firmly established. Here, we demonstrate that Numb acts both in a localization-dependent and in a localization-independent manner. We have generated *numb* mutants that affect only the asymmetric localization of the protein during mitosis. We demonstrate that asymmetric segregation of Numb into one of the two daughter cells is absolutely essential for cell-fate specification in the *Drosophila* peripheral nervous system. Numb localization is also essential in MP2 neuroblasts in the central nervous system and during muscle development. Surprisingly, in dividing ganglion mother cells or during heart development, Numb function is independent of its ability to segregate asymmetrically in mitosis. Our results suggest that two classes of asymmetric cell division exist, each with different requirements for asymmetric inheritance of cell-fate determinants.

Results

Numb is a cell-cortex-associated protein. During asymmetric cell division, Numb concentrates in the cell-cortex area overlying one of the two spindle poles and is preferentially inherited by one of the two daughter cells [3, 8]. In *numb* mutants, this cell is transformed into its sister cell [2]. Conversely, *numb* overexpression leads to the opposite cell-fate transformation [3]. Numb acts as a suppressor of Notch signaling [9, 10]. Via its PTB domain, it can bind to Notch [9] and to Sanpodo, a transmembrane protein involved in Notch signaling ([11] and A. Hutterer and J.A.K., unpublished data). The C terminus binds to the endocytic protein α-Adaptin [12]. *α-adaptin* alleles lacking the Numb binding domain cause phenotypes similar to *numb* mutants, suggesting that Numb downregulates Notch by α-Adaptin-mediated endocytosis. When the PTB domain is deleted, Numb becomes completely nonfunctional [13]. Thus, the ability to inhibit Notch is essential for Numb to determine cell fates. Whether the asymmetric localization of Numb is important for cell-fate specification, however, is less clear. Mutant forms of Numb lacking the localization domain can still influence cell fates [14]. Furthermore, a mouse Numb homolog that is not asymmetric can partially rescue *Drosophila numb* mutants [15]. Finally, Numb can influence cell lineages even when mitosis is inhibited, suggesting that at least some of its functions are independent of cell division [16–18].

numb^{S52F} Specifically Affects Asymmetric Localization

To address the importance of Numb localization, we rescreened a collection of mutants affecting asymmetric cell division in *Drosophila* external sensory (ES) organs for defects in Numb localization. We identified one mutant that fails to complement a numb null allele, numb^{15}, and a deletion in the *numb* locus [Df (2L) 30AC]. Immunofluorescence of mutant imaginal-disc clones (Figures 1A–1A″) or embryos homozygous for the *numb*^{S52F} mutation (data not shown) show that it is present at wild-type levels and that protein stability or translation are unaffected. During asymmetric cell division, however, the mutant protein fails to localize asymmetrically and segregates into both daughter cells (Figures 1C–1H′). Sequence analysis reveals that nucleotide 155 of the *numb* open reading frame is changed from C to T, resulting in a Serine-to-Phenylalanine change at position 52 of the protein. The affected amino acid is in a region that was previously implicated in the asymmetric localization of Numb [14]. To test whether the mutation is responsible for the localization defect, myc-tagged Numb and Numb^{S52F} were expressed in transgenic flies from the *hsp70* promoter (*hs-numb-myc* and *hs-numb*^{S52F}*-myc).* Both proteins are expressed at similar levels (Figure 1B) and localize to the cell cortex. Whereas Numb-myc localizes into a basal cortical crescent in dividing neuroblasts (Figures 1I and 1I′), Numb^{S52F}-myc remains uniformly cortical (Figures 1J and 1J′) and is found in both daughter cells. Thus, Numb^{S52F} affects the asymmetric localization of Numb.

To test whether Numb^{S52F} retains the ability to suppress Notch, we expressed myc-tagged wild-type and mutant proteins in sensory-organ precursor (SOP) cells

*Correspondence: juergen.knoblich@imba.oeaw.ac.at

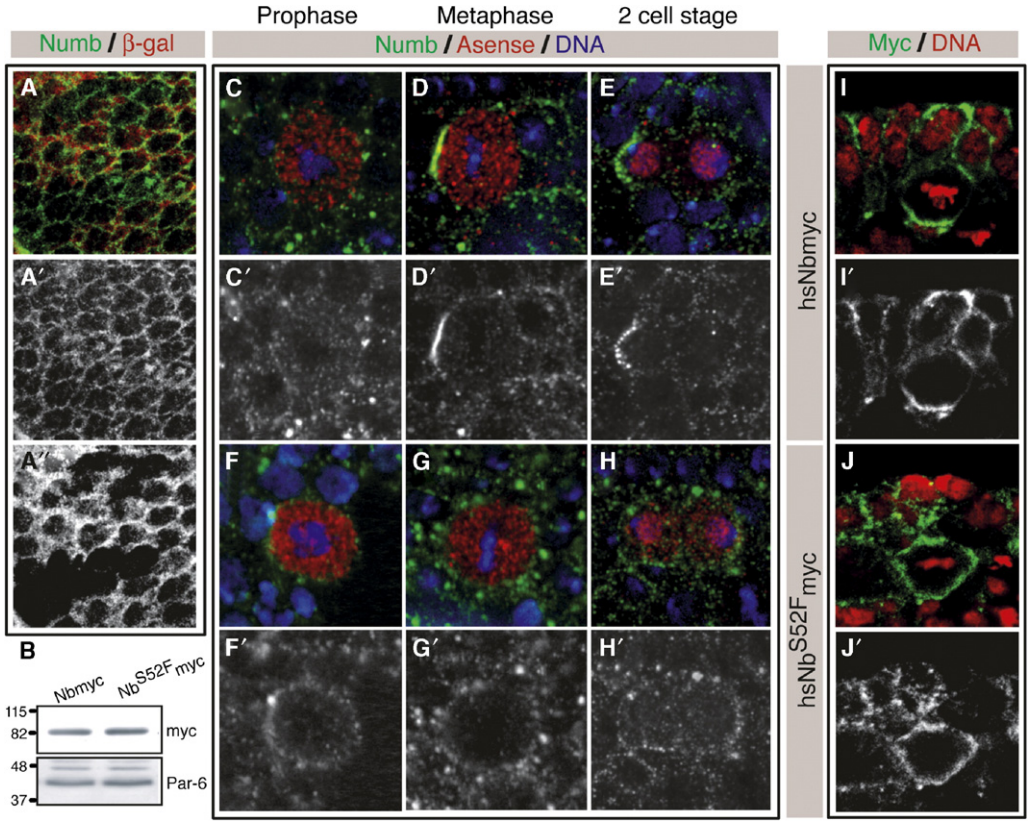

Figure 1. A Mutation in the Numb Localization Domain Affects Asymmetric Segregation

(A–A″) Clones of *numb*^{S52F} in eye imaginal disc stained for Numb (green, β-gal in red) (A) show wild-type levels of Numb expression (A′) in mutant clones marked by the absence of anti-β-gal staining (A″).

(B) *hs numb-myc* and *hs numb*^{S52F}*-myc* embryos express comparable amounts of myc-tagged protein. Loading control is Par-6.

(C–H′) In control SOP cells, Numb (green, DNA in blue, Asense—a SOP marker—in red) is cortical at prophase (C and C′) and then localizes asymmetrically (D and D′) and is inherited by the pIIb cell (E and E′). In *numb*^{S52F} clones, Numb is cortical at prophase (F and F′) but fails to form a crescent in metaphase (G and G′) and is inherited by both daughter cells (H and H′).

(I–J′) *hs numb-myc* (I and I′) and *hs numb*^{S52F}*-myc* (J and J′) embryos stained for myc (green) and DNA (red). Numb-myc localizes asymmetrically in dividing neuroblasts, whereas Numb^{S52F}-myc does not.

of the adult peripheral nervous system (PNS). SOP cells undergo a series of asymmetric cell divisions to generate the four different cell types found in external sensory (ES) organs (Figures 2A and 2E′). During each of these divisions, Numb segregates into one of the two daughter cells. In *numb* mutants, all divisions become symmetric, and four socket cells are generated. Upon Numb overexpression, SOP cells give rise to four neurons (Figure 2A) as a result of inhibition of Notch signaling in all cells of the lineage. Identical cell-fate transformations are observed when *hs-numb-myc* is expressed (Figures 2C and 2C′), indicating that a C-terminal myc tag does not affect the ability of Numb to inhibit Notch. When the identical heat-shock protocol is used to express *hs-numb*^{S52F}*-myc*, cell-fate transformations are observed with comparable frequency (Figures 2D and 2D′). Thus, Numb-myc and Numb^{S52F}-myc are similar in their ability to inhibit Notch. Consistent with this, the wild-type and mutant proteins coimmunoprecipitate similar amounts of Sanpodo and α-Adaptin (Figure 2B). S52F also did not affect binding to Partner of Numb (PON) (Figure 2B), another known binding partner of Numb [19]. Although Numb^{S52F} still binds PON, the asymmetric localization of PON in mitotic SOPs is unaffected in *numb*^{S52F} (see Figure S1 in the Supplemental Data available with this article online). This is actually surprising because it means that the interaction with asymmetrically localized PON is not sufficient for Numb to localize asymmetrically. We conclude that the S52F mutation does not affect Notch inhibition or interaction with any of the known in vivo binding partners of *Drosophila* Numb. Although we cannot exclude that other, unknown binding partners exist, these data suggest that *numb*^{S52F} specifically affects the asymmetric segregation of the Numb protein during mitosis. Consistent with this, *numb*^{S52F} (unlike other *numb* loss-of-function alleles) has no phenotype in a *Cyclin A* mutant background where mitosis is blocked (see Numb Localization Is Dispensable in Ganglion Mother Cells). Furthermore, the cell-fate transformations we observe (see Table 1) do not allow the placement of *numb*^{S52F} into an allelic series and indicate that *numb*^{S52F} is not simply a hypomorphic allele. We therefore used *numb*^{S52F} to address the rele-

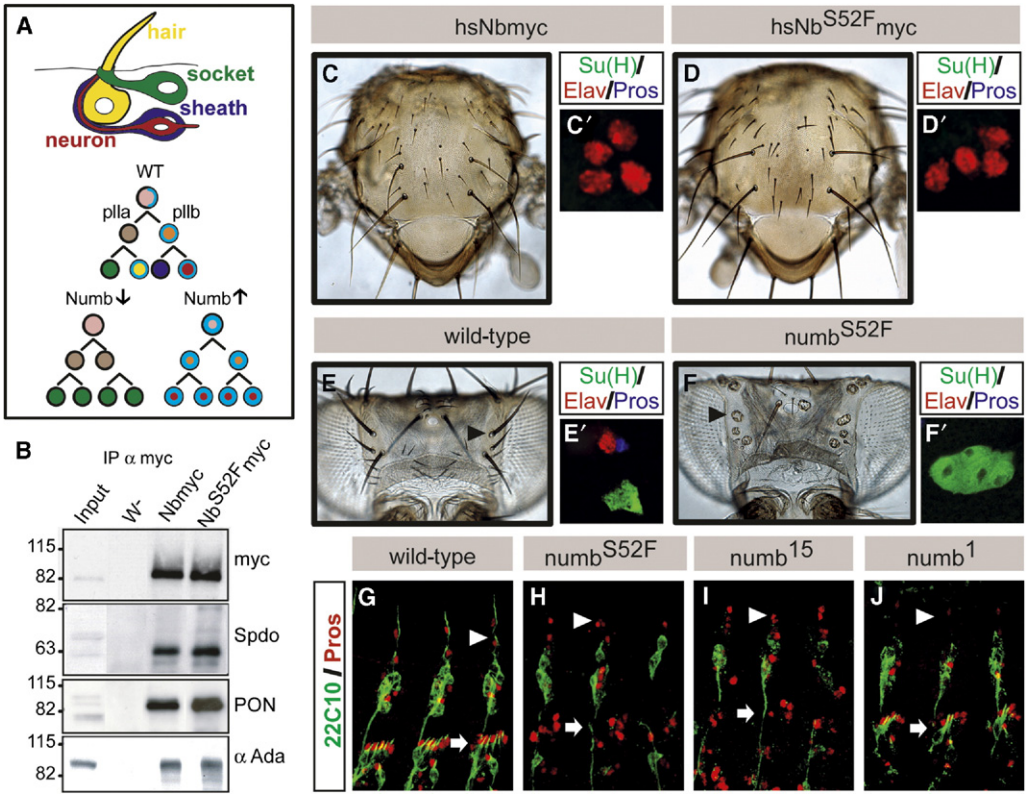

Figure 2. Numb^{S52F} Can Suppress Notch but Causes Cell-Fate Transformations in ES Organs

(A) ES-organ lineage in wild-type, *numb* mutant, and *numb*-overexpressing flies; Numb protein is in blue. An additional glia cell that does not contribute to ES organs is left out for simplicity.

(B) Anti-myc IPs from control *w-*, *hs numb-myc*, and *hs numb^{S52F}-myc* embryos probed for myc, Sanpodo, α-adaptin, and PON. Input is 1/100th of total extract from *hs numb^{S52F}-myc* used in the experiment.

(C) Nota of *hs numb-myc* flies show balding due to formations of an ES organ with four neurons (Elav, red) (C′).

(D) *hs numb^{S52F}-myc* flies show similar cell-fate transformations (D′). Note that SOP cell loss is observed frequently in (C) and (D) as well as upon overexpression of untagged *numb* (not shown).

(E) On wild-type *Drosophila* heads, hairs and sockets are visible as distinct morphological structures (arrowhead). Lineage staining of ES organs in pupae (E′) shows one socket (Suppressor of Hairless, green), one sheath (Prospero, blue), and one neuron (Elav, red).

(F) In *numb^{S52F}* mutant clones, most ES organs contain four socket cells (arrowhead), which express Su(H) (F′).

(G–J) PNS of wild-type (G) and mutant embryos (stage 14–15) stained with 22C10 (green) and Pros (red). *numb^{S52F}* embryos (H) show a loss of neurons in the dorsal ES organs (arrowheads) and the lateral chordotonal organs (arrow); the loss is similar to that in *numb^{15}* (I) but stronger than that in *numb^{1}* (J).

Table 1. Quantification of Cell-Fate Transformations in Zygotic *numb* Mutant Embryos

Lineage	Transformation	*numb^{15}* (n)	*numb^{S52F}* (n)	*numb^{3}*	*numb^{1}* (n)	Allelic Series
MP2	dMP2 → vMP2	100% (32)	100% (32)	n.d.	100%*	*nb^{15} = nb^{S52F} = nb^{1}*
GMC4-2a	RP2 → RP2sib	45.12% (82)	5.9% (119)	2%*	2.02% (99)	*nb^{15} > nb^{S52F} ≥ nb^{3} ≥ nb^{1}*
P15	FDA1 → sib	100% (10)	87.3% (41)	96%*	73%*	*nb^{15} > nb^{3} > nb^{S52F} > nb^{1}*
P17	FDO1 → sib	100% (100)	72% (43)	51.2%*	26.27%*	*nb^{15} > nb^{S52F} > nb^{3} > nb^{1}*
P2	FDO2 → FEPC	100% (9)	0% (37)	n.d.	80%*	*nb^{15} > nb^{1} > nb^{S52F}*
SOP (embryonic PNS)	absence of all neurons	54.16% (n = 24)	51.42% (35)	n.d.	10% (48)	*nb^{15} = nb^{S52F} > nb^{1}*
GMC4-2a (*Cyclin A* mutant background**)	RP2 → RP2sib	85% (n = 116)	1% (96)	n.d.	37.5% (104)	*nb^{15} > nb^{1} > nb^{S52F}*

Lineages derived from the MP2 neuroblast, GMC4-2a, muscle precursors P15, P17, and P2, and neurons in the embryonic PNS were analyzed. Numbers indicate the fraction of hemisegments displaying the indicated cell-fate transformations; (n) is the number of hemisegments analyzed. * shows the published *numb^{3}* and/or *numb^{1}* phenotypes in the MP2 [10], GMC4-2a [20], P15 and P17 [6], and P2 [18] lineages. ** GMC4-2a fate was analyzed in *Cyclin A* or *numb; Cyclin A* mutants where GMC4-2a does not divide and becomes an RP2 neuron or a RP2sib cell, respectively.

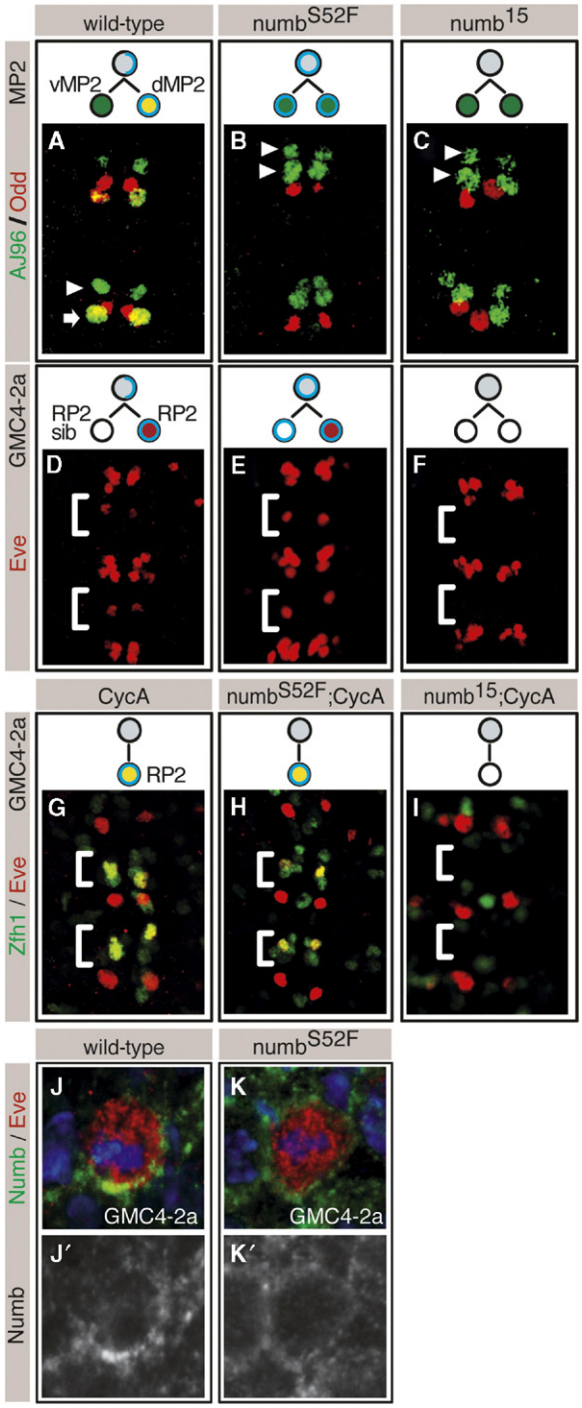

Figure 3. Numb Asymmetry Is Required in MP2 but Not in GMC4-2a

(A–C) CNS of wild-type and mutant embryos (stage 14–15) in AJ96 (β-gal) background, double labeled with anti-β-gal (green) and anti-Odd (red). In wild-type embryos (A), dMP2 (arrow) and vMP2 (arrowhead) express β-gal, whereas Odd marks only the dMP2 and the lineally unrelated MP1 neuron. In *numb*^{S52F} (B) and *numb*^{15} (C) mutants, both daughter cells are Odd negative, indicating a dMP2-to-vMP2 transformation (arrowheads).

(D–F) CNS of stage 15 embryos stained for Eve (red). RP2 neurons are in brackets. In wild-type (D) and maternal and zygotic *numb*^{S52F} mutant (E) embryos, Eve is maintained in RP2 (within bracket) but not RP2sib. In maternal and zygotic *numb*^{15} mutants (F), RP2 takes on the RP2sib fate and is undetectable in 100% of n = 124 hemisegments.

(G–I) CNS of stage 14–15 embryos stained for Eve (red) and Zfh-1 (green, marks RP2 but not GMC4-2a). In *Cyclin A* mutants, GMC4-2a division is blocked, but RP2 fate is established and Zfh-1 is on (G). In *numb*^{S52F}*; Cyclin A* double mutants (H) all GMC4-2a cells become RP2 neurons, indicating that Numb^{S52F} is fully functional when mitosis is blocked. Note that 1.5% of hemisegments had to be disregarded because RP2 was too close to CQ neurons. In *numb*^{15}*; Cyclin A* double mutants (I), most GMC4-2a cells become RP2siblings and switch off Eve expression.

(J–K′) Numb localization in GMC4-2a. Whereas in wild-type GMC4-2a, Numb (green, DNA in blue, Eve in red) localizes asymmetrically (J and J′), in *numb*^{S52F} mutants, it fails to form a crescent at metaphase (K and K′).

vance of Numb localization for asymmetric cell division in various tissues.

Numb Localization Is Essential in External Sensory Organs and MP2 Neuroblasts

In *numb* loss-of-function mutants, SOP cells undergo two rounds of symmetric division and generate four socket cells. This phenotype is dosage sensitive and is only observed at high penetrance in *numb* null alleles, like *numb*^{15}. Surprisingly, in contrast to Numb overexpression, in *numb*^{S52F} mutant clones, all ES organs (n = 54) show cell-fate transformations similar to those in *numb*^{15}. In 90.7%, four socket cells are present (Figures 2F and 2F′), whereas 7.4% have two sockets, one neuron, and one sheath (only one of the second divisions is affected), and 1.8% have two hairs and two sockets (only the first division is affected). Strong loss-of-function phenotypes are also observed in the embryonic PNS: In *numb*^{S52F} mutant embryos (Figure 2H), 52% (n = 35) of all hemisegments show a complete loss of all sensory neurons. This is similar to the null allele *numb*^{15} (Figure 2I) (neurons absent in 54% (n = 24) of all hemisegments) but is different from the hypomorph *numb*^{1} (Figure 2J) (10% of n = 48 hemisegments), indicating that *numb*^{S52F} behaves like a null allele during embryonic PNS development. In embryos both maternally and zygotically mutant for *numb*^{S52F}, almost all hemisegments (86%, n = 65) show a loss of all sensory neurons, and this is similar to embryos mutant for maternal and zygotic *numb*^{15} (92%, n = 55).

The *Drosophila* central nervous system arises from precursors called neuroblasts. During neuroblast division, Numb segregates asymmetrically but is not required for correct specification. Neuroblast divisions give rise to an apical daughter cell that retains neuroblast characteristics and a basal ganglion mother cell (GMC), which divides one more time to form two differentiating neurons or glia. One exception to this rule is the midline neuroblast MP2, which does not generate a GMC but rather divides to give a dorsal dMP2 and a ventral vMP2 neuron. Both dMP2 and vMP2 are labeled by β-gal expressed from the enhancer trap line AJ96, whereas only dMP2 inherits Numb and maintains expression of the transcription factor Odd (Figure 3A) [4]. In *numb*^{15} mutants, two vMP2s are generated (Figure 3C). Similarly, in *numb*^{S52F} mutants, 100% of the MP2 neuroblasts show the *numb* loss-of-function phenotype and give rise to two vMP2 cells (Figure 3B). These data suggest that asymmetric localization of Numb is impor-

tant for cell-fate specification in SOP cells and the MP2 neuroblast.

Numb Localization Is Dispensable in Ganglion Mother Cells

Numb is also involved in asymmetric division of GMCs. In wild-type embryos, GMC4-2a localizes Numb asymmetrically (Figures 3J and 3J′) and divides into an RP2 neuron that continues to express Even-skipped (Eve) and a sibling cell (RP2sib) that downregulates Eve shortly after mitosis (Figure 3D) [20]. Numb enters the RP2 and in strong *numb* alleles, two RP2sib cells are generated (Figure 3F) [20] that extinguish Eve expression. We analyzed the GMC4-2a lineage in $numb^{S52F}$ mutants. Although $Numb^{S52F}$ segregates into both daughter cells (Figures 3K and 3K′), in $numb^{S52F}$ maternal and zygotic mutant embryos, 95% of the hemisegments still have only one Eve-expressing daughter cell at the position of RP2, indicating that asymmetric cell division was unaffected (Figure 3E). This suggests that asymmetric localization is not strictly required for Numb function during GMC4-2a division. Although this result is surprising, it is consistent with previous observations that postulate a segregation-independent function for Numb [16, 17]. When cell division is blocked in *Cyclin A* mutants, GMC4-2a differentiates into one RP2 neuron (n = 152, Figure 3G). In $numb^{15}$*; Cyclin A* double mutants, however, in 85% of the cases an RP2sib is generated (n = 116, Figure 3I). This adoption of an RP2sib fate, although to a lesser extent (37.5%, n = 104), is seen even in weak hypomorphic alleles of numb such as $numb^{1}$ [16, 17]. If $numb^{S52F}$ is specific for asymmetric localization and is not a hypomorph, we would expect that it is fully functional when mitosis is blocked. We therefore analyzed GMC4-2a in $numb^{S52F}$*; Cyclin A* double mutants. In these mutants, 99% of the GMC4-2a cells (n = 96) differentiate into an RP2 neuron (Figure 3H), thus demonstrating that $Numb^{S52F}$ has retained its Notch-suppressing activity. The absence of even a weak transformation of the GMC4-2a into the RP2sib (as it is observed in $numb^{1}$) indicates that $numb^{S52F}$ is not a hypomorph. This is further supported by the muscle phenotype of $numb^{S52F}$*; Cyclin A* double mutants (see below).

Importance of Numb Localization in Muscle Development

In addition to its role in the nervous system, Numb has a conserved function in muscle development [5–7, 21]. Numb localizes asymmetrically in the *Drosophila* muscle precursors P2 (Figures 4G–4H′), P15, and P17 and is required for correct specification of their daughter cells. We therefore addressed the importance of Numb localization in individual muscle lineages. P15 and P17 divide asymmetrically into one sibling cell of unknown fate and the founder cells FDA1 and FDO1, respectively, that inherit Numb [6]. Both founder cells later fuse with surrounding fusion-competent cells to form muscles DA1 and DO1. Whereas both P15 and P17 and the two founder cells express Krüppel (Kr), Eve is expressed only in P15 and FDA1 and can be used to distinguish the two lineages (Figure 4A). FDA1 can also be identified by staining for Eve and the muscle marker Mef2 (Figure 4D). In *numb* loss-of-function mutants, FDA1 and FDO1 are transformed into their sibling cells (Figures 4C and 4F) [6], which is confirmed by a loss of the corresponding muscles (Figure S1). Similar cell-fate transformations are observed in $numb^{S52F}$ mutants (Figures 4B and 4E), indicating that Numb function in muscle development is dependent on its asymmetric localization. To confirm that $Numb^{S52F}$ is able to repress Notch in the P15 and P17 lineages, we analyzed $numb^{S52F}$*; Cyclin A* double mutants. In *Cyclin A* mutants, P15 and P17 do not divide and assume the FDA1 and FDO1 fates, respectively (Figure 4K). In $numb^{1}$*; Cyclin A* double mutants, P15 takes the sibling cell fate [18]. In $numb^{S52F}$*; Cyclin A* double mutants, however, one FDA1 and one FDO1 are formed (Figure 4L), indicating that $Numb^{S52F}$ is fully functional when cell division is blocked.

In contrast to P15 and P17, the P2 precursor not only generates muscle cells but also gives rise to pericardial cells of the *Drosophila* heart. In wild-type embryos, the daughter cell that inherits Numb becomes the founder of muscle DO2 (FDO2), whereas its sibling cell (FEPC) divides once more into two pericardial cells (EPCs) [6, 18]. After division, Eve is maintained in the EPCs (Figure 4D) and is transiently present in the FDO2, which switches on the muscle marker Mef2. In *numb* mutants, P2 divides symmetrically and generates four EPCs (Figure 4F). Surprisingly, even though $Numb^{S52F}$ fails to localize asymmetrically (Figures 4I–4J′), no defects are seen in the P2 lineage of either maternal and zygotic (data not shown) or zygotic $numb^{S52F}$ mutants (Figure 4E), indicating that in this lineage, Numb functions in a way that does not require its asymmetric localization. This agrees with previous observations that P2, like GMC4-2a, requires Numb function for cell-fate specification even when cell division is blocked [18].

Discussion

Numb Localization Is Important for Asymmetric Cell Division

Previously, mutations in *bazooka* and *inscuteable* or *numb* overexpression have been used to analyze the importance of Numb localization during cell division. In all cases, the phenotypes observed are different from the $numb^{S52F}$ phenotype. In these mutants, localization of other cell-fate determinants is also affected, causing phenotypes not solely as a result of Numb mislocalization. Other biological processes like epithelial polarity, spindle positioning, and cell size [6, 20, 22] are affected in *bazooka* and *inscuteable* mutants and could be responsible for the observed phenotypes in these mutants. Numb localization is also inhibited upon overexpression of the protein, presumably as a result of saturation of the localization machinery. Upon overexpression, Numb is segregated into both daughter cells that then adopt the fate of the daughter that normally inherits *numb* [3]. In $numb^{S52F}$ mutants, however, we observe loss-of-function phenotypes in most of the lineages analyzed. Either $numb^{S52F}$ is a hypomorph that only partially retains its ability to suppress Notch, or, alternatively, asymmetric localization is crucial for Numb to act as a cell-fate determinant. We favor the second

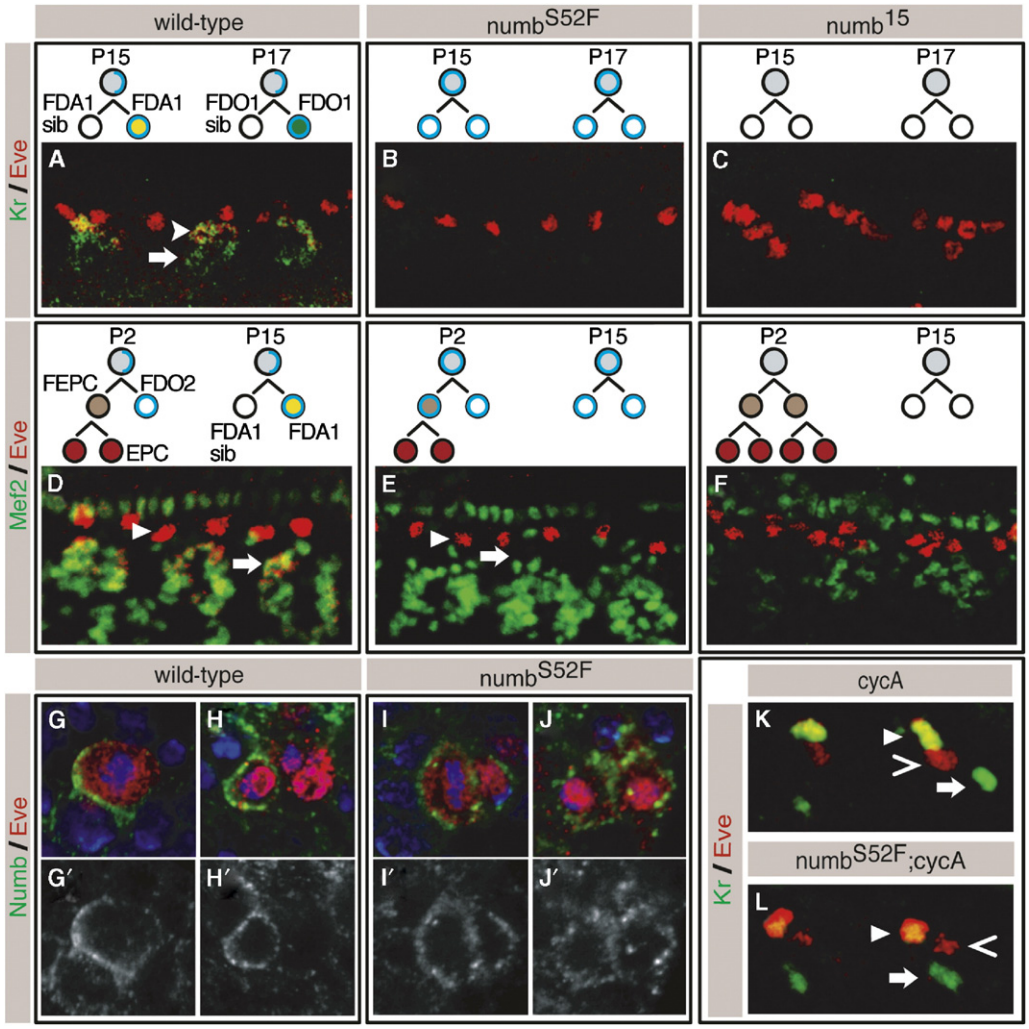

Figure 4. Loss of Numb Asymmetry Affects Muscle-Precursor Division but Not Pericardial-Precursor Division

(A–C) Three hemisegments of stage 14–15 embryos double stained for Eve (red) and Kr (green). In wild-type (A), DA1 precursors (FDA1, Eve$^+$, Kr$^+$, arrowhead) and DO1 precursors (FDO1, Kr$^+$, Eve$^-$, arrow) are seen. Note that staining is diffuse because muscle cell fusion has started. In *numb^{S52F}* mutants (B), the DA1 (>85% loss) and DO1 (>70% loss) muscle precursors are absent. In *numb^{15}* mutants (C), a similar phenotype is observed. Eve$^+$, Kr$^-$ cells are from the unrelated P2 lineage (see below, [F]).

(D–F) Three hemisegments of stage 14 embryos stained with Eve (red) and Mef2 (green). (D) In wild-type, two EPCs (red, arrowhead) that arise from the Eve+ P2 progenitor and the DA1 muscle (yellow, arrow) that arises from the P15 muscle precursor are seen. (E) In *numb^{S52F}* mutants, the P2 precursor lineage is wild-type because there are two EPCs (red, arrowhead) per hemisegment. However, the P15 lineage is affected, as seen by the loss of the DA1 muscle (arrow). (F) In *numb^{15}* mutants, the number of EPCs is increased to 3.8 per hemisegment, and the DA1 muscles are not formed.

(G–J′) Numb localization in dividing P2 precursors. In control P2 precursors, Numb (green, DNA in blue, Eve in red) (G) localizes asymmetrically (G′) and is inherited by the FDO2 cell (H and H′). In *numb^{S52F}* animals, Numb fails to form a crescent in metaphase (I and I′) and is inherited by both daughter cells (J and J′).

(K and L) Stage 13–14 embryos stained for Eve (red) and Kr (green). (K) When muscle-precursor division is blocked in *Cyclin A* mutants, P2, P15, and P17 take on the fate of FDO2 (open arrowhead), FDA1 (arrowhead), and FDO1 (arrow), respectively. (L) The identical phenotype is observed in *numb^{S52F}; Cyclin A* double mutants. Note that in (K) and (L), FDO2 has not yet downregulated Eve.

possibility for several reasons: First, Numb^{S52F} completely retains its ability to bind Sanpodo and α-Adaptin; second, *numb^{S52F}* is as potent as the wild-type protein in inducing cell-fate transformations upon overexpression; third, Numb^{S52F} is fully functional in inhibiting Notch when cell division is blocked in *Cyclin A* mutants; and finally, *numb^{S52F}* cannot be consistently placed into an allelic series (see Table 1). In the P2 lineage, for example, *numb^{S52F}* mutants are completely wild-type, whereas in the SOP lineage, they behave like null alleles. We therefore conclude that Numb^{S52F} is specific for asymmetric localization and that asymmetric localization is essential for Numb to act as a cell-fate determinant during one class of asymmetric divisions that occur in the SOP cells, MP2 neuroblasts, and muscle precursors.

Localization-Independent Functions of Numb

We show that two classes of asymmetric cell divisions can be distinguished. In class I divisions, Numb localization is essential for the two daughter cells to assume different fates. In these divisions, one cell takes on a

different fate only when Numb is concentrated in this cell. During class II divisions, however, Numb acts independently of its asymmetric segregation. In such divisions, which occur in GMCs and during heart development, one possibility would be that Numb functions via downstream effectors other than those involved in class I divisions. However, the Notch/Delta pathway is the downstream target of Numb function in these lineages as well [7, 10, 17, 18, 20]. Sanpodo, another gene acting downstream of Numb, is involved not only in divisions of the SOP, MP2, neuroblast, and muscle precursors but also during divisions of the P2 and GMC4-2a [7, 11, 18, 20, 23].

What could be the reason for the asymmetric outcome of these divisions? Other segregating determinants could exist that act redundantly with Numb during class II divisions but may not be present during class I divisions. Neuralized would be a candidate because it is the only other segregating determinant known to act on the Notch/Delta system [24]. However, Neuralized has only been shown to be essential for asymmetric cell division in ES organs. Because these divisions belong to class I, Neuralized is unlikely to be responsible for the asymmetric outcome of class II divisions. Alternatively, feedback loops in the Notch/Delta pathway could amplify small, random differences in Notch activity to establish distinct fates even when Numb concentrations are the same [25]. Finally, Numb could act redundantly with polarized extracellular signals that act differently on the two daughter cells.

Conclusions

We propose that Numb can act in both a localization-dependent and -independent way. Our results are of particular importance for vertebrates, where both localizing and nonlocalizing homologs of Numb are involved in nervous-system development [26]. It is conceivable that localization-dependent and localization-independent functions have separated into two distinct homologs during evolution. The existing data do not allow us to tell whether vertebrate neural-precursor divisions are of class I or class II. However, Serine 52 is conserved in mouse Numb [27, 28], and its targeted mutation could be used to address the relevance of Numb localization in vertebrates.

Experimental Procedures

Constructs, Flies, and Antibodies

numb^{S52F} clones were generated with the *eyless-flp*/FRT/cell-lethal system [29] or *Ubx-Flp*. *Ubx-Flp* was generated by inserting two copies of the *Ubx* enhancer fragment PBX-41 (gift from M. Bienz) into *pCaSpeR-hsFlp,* which carries the Flp recombinase under control of a complete *hsp70* promoter. *Ubx-Flp* induces recombination in all imaginal discs. Numb germline clones were generated with *ovo*^{D1} and *hs-Flp* in a similar manner as described [30]. For *numb* overexpression, full-length *numb* or *numb*^{S52F} with nine C-terminal myc tags were cloned into *pCaSpeRhs.* Expression in embryos was performed by heat shocking 3–6-hr-old embryos for 30 min at 32°C, followed by recovery at 25°C for 30 min. Expression in pupae was at 39°C for 45 min at 16–18 hr APF. Other fly strains were *CycA*^{C8LR1} (from Christian Lehner) and AJ96 (from Christian Klaembt). Homozygous embryos were identified by β-gal-expressing balancers. The following antibodies were used: rabbit anti-Numb (1:100) [31], rabbit anti-Eve (from M. Frasch, 1:2000), rabbit anti-α-ada (1:100) [12], rabbit anti-Pros (from Y.N. Jan, 1:1000), rabbit anti-Odd (from J. Skeath, 1:400), rabbit anti-Kr (from H. Jaeckle, 1:200), rabbit anti-Mef2 (from H. Nguyen,1:600), rabbit anti-β-gal (Cappel, 1:3000), rabbit anti-PON (from Y.N. Jan, 1:1000), rabbit anti-β3-tubulin (from R. Renkawitz-Pohl, 1:700) mouse anti-Numb (1:200) [32], mouse anti-c-*myc* (9E10, 1:100), mouse anti-β-gal (Promega, 1:100), mouse anti-Elav (DSHB mAB9F8A9, 1:30), mouse anti-Futsch (DSHB mAB22C10, 1:150), mouse anti-Eve (DSHB mAB2B8, 1:50), mouse anti-DmPar-6 (1:100) [33], mouse anti-Zfh1 (from Z. Lai, 1:500), rat anti-Su(H) (from F. Schweisguth, 1:2000), guinea pig anti-Ase (1:1000, against the peptide CLSDESMIDAIDWWEAHAPKSN GACTNLSV), and guinea pig anti-Spdo (1:1000 generated against N-terminal MBP fusion protein). Images were recorded on a Zeiss LSM510 confocal microscope.

Protein-Binding Assays

Transgene expression was induced by heat shocking 3–6-hr-old embryos for 30 min at 37°C, followed by recovery at 25°C for 30 min. Protein extracts were prepared in extraction buffer (25 mM Tris [pH 8], 27.5 mM NaCl, 20 mM KCl, 25 mM Sucrose, 10 mM EDTA, 10% (v/v) glycerol, 1 mM DTT, and 0.1% NP40). Immunoprecipitations were carried out with anti-myc antibody (prepared from cell line 9E10) for 3 hr at 4°C. Washes were 3 × 5 min and 3 × 15 min.

Supplemental Data

Supplemental Data include one supplemental figure and are available with this article online at http://www.current-biology.com/cgi/content/full/15/17/1583/DC1/.

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